THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 252, No. 9, Issue of May 10, pp. 2808-2814, 1977
Printed in U.S.A.

A New Endonuclease from *Escherichia coli* Acting at Apurinic Sites in DNA*

(Received for publication, November 11, 1976)

SIV LJUNGQUIST

From the Department of Chemistry, Karolinska Institute, 104 01 Stockholm, Sweden

A new DNA endonuclease has been purified 3000-fold from *Escherichia coli*. The enzyme specifically catalyzes the formation of single strand breaks at apurinic and apyrimidinic sites in DNA, but has no activity on intact or single-stranded DNA. Further, the enzyme shows little or no activity on heavily ultraviolet-irradiated DNA, but cleaves x-irradiated DNA, presumably at apurinic and apyrimidinic sites introduced by the radiation treatment. The enzyme, which is tentatively named endonuclease IV, has no detectable associated exonuclease or DNA N-glycosidase activity and does not seem to be identical with any previously known *E. coli* endonuclease. Endonuclease IV has no Mg^{2+} requirement, and is fully active in the presence of EDTA. Enzyme activity is stimulated by 0.2 to 0.3 M NaCl and is unusually salt-resistant. Further, the enzyme is fairly heat-stable, and is not inhibited by tRNA. The sedimentation coefficient, $s^{\circ}_{20,w}$, is 3.4 S. It seems likely that endonuclease IV is active in DNA repair.

Endonucleases that specifically introduce single strand breaks at apurinic sites in DNA have been isolated from many sources, including *Escherichia coli* (1). The main enzyme activity of this kind in *E. coli* cell extracts has been shown to be identical with a previously known *E. coli* nuclease, DNA ex nuclease III (2, 3). It would appear that this enzyme acts as a DNA exonuclease and 3'-phosphatase, and in addition causes incisions at apurinic sites in DNA.

Several *E. coli* mutants defective in this major endonuclease activity for apurinic sites have recently been isolated (4–6). Such mutants, *xthA* , are slightly more sensitive to methyl m thanesulfonate than wild type *E. coli*, indicating that they are DNA repair-defective. Crude cell extracts of *xthA* mutants still contain about 10% of the normal level of endonuclease activity for apurinic sites in DNA. This residual activity differs in EDTA sensitivity and heat stability from the endonuclease function of exonuclease III, and was found to be present in similar amounts in wild type strains and in several *xthA* mutants, including an *xthA* deletion mutant (5). Thus, th activity clearly depended on ne or several enzymes different from exonuclease III. Most of this activity on DNA containing apurinic sites seems to be due to a new enzym , tentatively called *E. coli* DNA endonuclease IV, as it does not have detectable associated exonuclease activity. The present paper describes the purification and characterization of endonuclease IV.

* This work was supported by a grant from the Karolinska Institute, and by grants to T. L. from the Swedish Natural Science Research Council and the Swedish Cancer Society.

MATERIALS AND METHODS

Nucleic Acids

Escherichia coli B/r [^{32}P]DNA (20,000 cpm/μg) was prepared according to the method of Grossman (7) and phage T7 [^{3}H]DNA (56,000 cpm/μg) according to the method of Richardson (8). Phage PM2 [^{32}P]DNA (3,600 cpm/μg measured as Cerenkov radiation in H_2O) which has a covalently closed circular duplex structure, was made according to the method of Masamune and Richardson (9). [^{3}H]Uridine-labeled PBS-1 DNA (42,000 cpm/μg) was a gift from T. Lindahl, Karolinska Institute. Calf thymus DNA was purchased from Worthington Biochemical Co. *E. coli* tRNA and yeast tRNA were made by standard methods (10).

For the preparation of depurinated alkylated DNA (1), *E. coli* [^{32}P]DNA (50 μg/ml) in 0.05 M potassium phosphate, pH 7.4, was treated with 0.3 M methylmethanesulfonate at 37° for 45 min. Remaining methylmethanesulfonate was then removed by dialysis against 0.2 M NaCl, 0.01 M sodium citrate, 0.01 M sodium phosphate, pH 7.0, at 2° for 16 h. The alkylated DNA was incubated at 50° for 6 h to release methylated purine residues. The partly depurinated DNA was dialyzed against 0.1 M NaCl, 0.01 M sodium citrate, pH 6.0, at 2° for 20 h, and was stored in several small aliquots at −70°.

Phage DNAs containing small numbers of apurinic sites were prepared by brief incubation at 70° in 0.1 M NaCl, 0.01 M sodium citrate, pH 5.0. Under these conditions, one apurinic site per PM2 DNA molecule was introduced in 7 min (11).

Ultraviolet-irradiated DNA was made essentially as previously described (12). PM2 [^{32}P]DNA (9 μg/ml in 0.015 M NaCl, 0.0015 M trisodium citrate, pH 7.0) was irradiated with a precalibrated, low pressure mercury lamp. After irradition of the DNA, 0.05 volume of 2 M NaCl was added. Heat treatment of a part of the irradiated DNA was then performed by incubation at 70° for 30 min.

X-irradiated PM2 DNA was obtained by irradiation of the DNA (17 μg/ml in 0.1 M NaCl, 0.02 M Tris/HCl, 10^{-3} M EDTA, pH 7.1) under air, with the DNA solution in a small chilled Petri dish. A Siemens roentgen unit was the source of 190 kV x-rays filtered with aluminium, and the dose rate was 350 rads/min. After irradiation, 0.05 volume of 2 M NaCl was added, and one-half of the DNA solution was kept at 0°, while the other half was incubated at 70° for 30 min. Irradiation of the DNA, subsequent incubation with enzyme, and analysis by gradient centrifugation were always performed on the same day.

Reagent Enzymes and Other Materials

E. coli alkaline phosphatase, bovine pancreatic DNase I, bovine catalase, and egg white lysozyme were purchased from Worthington, and bovine carbonic anhydrase and ovalbumin from Sigma. *Micrococcus luteus* ultraviolet endonuclease was prepared according to the method of Kaplan *et al.* (13) up to the DNA-cellulose chromatography step. *E. coli* thioredoxin was a gift from A. Holmgren, Karolinska Institute.

Ammonium sulfate, special enzyme grade, was purchased from

Schwarz/Mann; Hepes,[1] and *p*-hydroxymercuribenzoate from Sigma; and Sephadex G-75, Sephadex G-100, and blue dextran from Pharmacia. Native DNA-cellulose containing calf thymus DNA was prepared according to the method of Alberts and Herrick (14), and hydroxyapatite according to the method of Bernardi (15).

Analytical Procedures

Centrifugations in linear 5 to 20% sucrose gradients were done at 40,000 rpm and 5° in a Spinco SW 41 rotor. Neutral gradients contained 0.02 M sodium phosphate, 10^{-3} M EDTA, pH 7.0, while alkaline gradients contained 0.5 M NaCl, 0.3 M NaOH, 0.01 M EDTA. PM2 DNA was centrifuged for 6 h in neutral gradients and 3 h in alkaline gradients, while T7 DNA was centrifuged for 5 h in both types of gradients. Fractions were collected from the bottoms of the tubes. Prior to analysis in alkaline gradients, all DNA solutions were mixed with 1 volume of 0.4 M NaOH and incubated at 20° for 15 h to cleave the chains at apurinic and apyrimidinic sites (11).

The molecular weight and frictional ratio of the endonuclease were evaluated from its sedimentation coefficient and Stokes radius according to the method of Siegel and Monty (16), assuming a partial specific volume of the protein of 0.725 cm^3/g (17). The reference compounds were alkaline phosphatase, carbonic anhydrase, and lysozyme in sucrose gradients, and blue dextran, catalase, alkaline phosphatase, ovalbumin, pancreatic DNase, carbonic anhydrase, thioredoxin, and [^{14}C]leucine in gel filtration experiments employing Sephadex G-100.

Endonuclease Assay

The assay is based on the release of acid-soluble material from heavily depurinated, radioactive DNA (1, 18). The standard reaction mixture (0.1 ml) contains 0.2 M NaCl, 0.05 M Hepes·KOH, pH 8.2, 10^{-3} M EDTA, 10^{-4} M dithiothreitol, 1 μg of depurinated alkylated *E. coli* [^{32}P]DNA, 50 μg of bovine serum albumin, a limiting amount of enzyme (2 to 20 units). After incubation at 37° for 30 min, the reaction mixtures are chilled, 0.1 ml of 0.8 M perchloric acid is added, acid-insoluble material is removed by centrifugation at 15,000 × *g* for 10 min, and the radioactivity of a 100-μl aliquot of the supernatant solution is determined. One enzyme unit, originally defined in a more time-consuming transformation assay (5, 19), releases 20 pmol of ^{32}P in an acid-soluble form from the DNA substrate under these conditions.

In reaction mixtures containing bacteriophage DNA instead of *E. coli* DNA, bovine serum albumin was excluded, Hepes·NaOH was used instead of Hepes·KOH, and the incubation time at 37° was reduced to 15 min. Reactions were stopped by chilling and addition of 0.1 volume of 2% sodium dodecyl sulfate prior to analysis by sucrose gradient centrifugation.

Enzyme Purification

Growth of Bacteria – *E. coli* 1100 (*endA*⁻) were grown in a glucose-salts medium supplemented with 0.2% casamino acids and 0.1% yeast extract, and were harvested in the logarithmic growth phase. The bacteria were stored at −70°, and disintegrated in a modified Hughes press at −25°.

Crude Cell Extract – Disintegrated bacteria (80 g) were thawed and stirred with 300 ml of 0.05 M Tris/HCl, pH 8.0, for 30 min, and the debris was removed by centrifugation (Fraction I).

All operations were performed at 0–4°. All buffers contained 10^{-3} M dithiothreitol. Centrifugations were at 20,000 × *g* for 15 min. Protein concentrations were determined by the biuret reaction (Fractions I to III) or by A_{280} measurements (Fractions IV and V).

Streptomycin Treatment and Ammonium Sulfate Fractionation – To Fraction I, 1 volume of 1.6% (w/v) streptomycin sulfate in 0.05 M Tris/HCl, pH 8.0, was slowly added under gentle stirring. After 1 h, the precipitate was removed by centrifugation. To the supernatant solution (620 ml), 164 g of ammonium sulfate was added and slowly dissolved. The precipitate formed was discarded after centrifugation. An additional 85 g of ammonium sulfate was added to the supernatant and slowly dissolved, and the precipitate was collected by centrifugation. It was dissolved in 50 ml of 0.2 M NaCl, 0.05 M potassium phosphate, pH 7.4, and dialyzed for 3 to 4 h against the same buffer (Fraction II).

Gel Filtration – Fraction II was applied to a column (4 × 117 cm) of Sephadex G-75 equilibrated with 0.2 M NaCl, 0.05 M potassium phosphate, pH 7.4, and eluted with the same buffer. Endonuclease IV was eluted after most of the protein, and active fractions were pooled (Fraction III).

Heat Treatment – Fraction III was treated at 65° for 5 min. The heating step was performed with 5-ml portions of the enzyme solution in 20-ml test tubes, with a precalibrated thermometer in one of the tubes. The rack of tubes was immersed in a 65° water bath, and the 5 min incubation period was started when the protein solution was within 0.5° of 65°. The heat treatment was stopped by rapid chilling in an ice bath. After 30 min at 0°, precipitated protein was removed by centrifugation, and the supernatant solution was dialyzed against 0.02 M Tris/HCl, pH 8.0, for 10 h (Fraction IV).

DNA-cellulose Chromatography – Fraction IV was applied to a column (2.2 × 7.5 cm) of native DNA-cellulose, equilibrated with 0.02 M Tris/HCl, pH 8.0, 5% glycerol. The column was washed with 100 ml of the same buffer, and endonuclease IV was eluted with 0.3 M NaCl, 0.02 M Tris/HCl, pH 8.0, 5% glycerol. Active fractions were pooled and applied to a DEAE-cellulose column (2 × 2 cm) equilibrated with 0.3 M NaCl, 0.02 M Tris/HCl, pH 8.0, 5% glycerol. Endonuclease IV was not adsorbed and was recovered in the effluent, while traces of DNA were removed. Active fractions were again pooled, concentrated about 10-fold with a Diaflo ultrafiltration apparatus, dialyzed against 0.3 M NaCl, 0.02 M Tris/HCl, pH 7.5, 5% glycerol for 20 h, mixed with 1 volume of glycerol, and stored frozen in several small aliquots at −70° (Fraction V). In this form, the enzyme shows no loss of activity after 6 months. An alternative concentration procedure was also employed. The DNA-cellulose eluate was supplemented with 0.02 volume of 0.5 M potassium phosphate, pH 7.5, and applied to a column (1.1 × 2 cm) of hydroxyapatite equilibrated with 0.3 M NaCl, 0.02 M Tris/HCl, 0.01 M potassium phosphate, pH 7.5. After extensive washing of the column with the same buffer, endonuclease IV was eluted with 0.3 M NaCl, 0.3 M potassium phosphate, pH 7.5. The eluate was mixed with 1 volume of glycerol and stored at −70°. The latter procedure gave lower recoveries of endonuclease activity (30 to 40% recovery) but removed trace quantities of uracil-DNA glycosidase present in Fraction V, as that enzyme does not adsorb to hydroxyapatite under the conditions used (20).

A summary of the purification procedure is given in Table I. Fraction V of endonuclease IV was used in all experiments described. Fraction V is approximately 3,000 times purified, but it is not a homogeneous enzyme, as five to six different protein bands have been observed in sodium dodecyl sulfate polyacrylamide slab gel electrophoresis experiments. Although endonuclease IV shows no Mg^{2+} requirement in the standard enzyme assay (see below), decreased yields, and a decreased heat stability of the enzyme are observed if EDTA (10^{-3} M) is included in the buffers during the enzyme preparation.

RESULTS

General Properties of Purified Enzyme – Endonuclease IV has a broad pH optimum at pH 8.0 to 8.5 in Hepes·KOH buffer and shows 50% of the optimal activity at pH 7.5. There is no detectable stimulation or inhibition of the enzyme activity when the EDTA in the standard reaction mixture is replaced by $MgCl_2$ (5×10^{-4} to 10^{-2} M) or 10^{-3} M $CaCl_2$ (Table II, see also

TABLE I

Purification of DNA endonuclease IV from 80 g of Escherichia coli cells

The enzyme assay used in the present work is unreliable with crude cell extracts due to interference of other nucleases, and the data for Fraction I are based on separate determinations of the activities of Fractions I and II by a transformation assay (5).

Fraction	Volume	Total protein	Specific activity	Total activity
	ml	*mg*	*units/mg*	*units* 10^{-6}
I. Crude extract	327	7,780	300	2.3
II. Ammonium sulfate	61	2,690	608	1.64
III. Sephadex G-75	133	370	3,810	1.41
IV. Heat treatment	130	280	4,260	1.17
V. DNA-cellulose	2.2	0.22	1,060,000	0.48

[1] The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

BEST AVAILABLE COPY

Fig. 2). Higher concentrations of $CaCl_2$ are inhibitory. The enzyme activity is unusually resistant to the presence of NaCl in reaction mixtures, and retains 50% of maximal activity in 0.56 M NaCl (Fig. 1). This property of endonuclease IV may be a useful criterium to distinguish it from other *Escherichia coli* endonucleases. The enzyme is strongly inhibited by *p*-hydroxymercuribenzoate, and a reducing agent such as dithiothreitol appears necessary for optimal activity (Table II). On heating in 0.2 M NaCl, 0.05 M potassium phosphate, 10^{-3} M dithiothreitol, pH 7.4, endonuclease IV retains full activity after 5 min at 60°, while 80 to 90% of the activity is retained after similar heating at 65° but < 10% after heating at 80°. Thus, the enzyme is fairly heat-stable. There is no inhibition of endonuclease IV by *E. coli* tRNA or yeast tRNA (20 to 500 μg/ml), nor is there any stimulation or inhibition by ATP (Table II).

The sedimentation coefficient of endonuclease IV is 3.4 S, as determined by sucrose gradient centrifugation together with alkaline phosphatase (6.3 S), bovine carbonic anhydrase (3.06 S), and egg white lysozyme (2.11 S). The Stokes radius of the enzyme was determined by analytical gel filtration to be 25 Å. These data indicate that endonuclease IV is a globular protein of little or no asymmetry, with a molecular weight of 33,000 ± 3,000 and a frictional ratio, f/f_o, of 1.18.

Endonuclease IV has been partly purified and characterized from several *E. coli* strains, including AB1157 (*endA*$^+$), AB1386 (*uvrA*$^-$), NH5016 (*xthA*$^-$), and BW9101 (*xthA*$^-$). The enzyme is present in similar amounts in these strains, and no *E. coli* strain deficient in endonuclease IV has so far been found.

Absence of DNA Exonuclease and Phosphatase Activity—*E. coli* exonuclease III can act as an exonuclease at the 3′ termini of double-stranded DNA, as a DNA 3′-phosphatase, and as an endonuclease at apurinic sites in DNA (2, 3). Endonuclease IV also acts as an endonuclease at apurinic sites in DNA, so it was of interest to search for an associated exonuclease or 3′-phosphatase activity. No release of acid-soluble material (< 0.1%) from native *E. coli* [^{32}P]DNA by endonuclease IV was observed in the standard reaction mixture or in a reaction mixture containing Mg^{2+} (Fig. 2). Similarly, Fraction III of the enzyme showed no detectable exonuclease activity on native DNA in the standard reaction mixture. Further, no exonuclease activity was observed with Fraction V when the DNA substrate was heat-denatured prior to use. Under the same conditions, release of acid-soluble material from partly depurinated DNA occurred, but a maximum of 15% of the total radioactivity could be released in the standard reaction mixture (Fig. 2). These data are similar to those previously obtained with a mammalian DNA endonuclease for apurinic sites, which lacks an associated exonuclease activity, but differ from those obtained with *E. coli* exonuclease III (18). Further, Fraction V of *E. coli* endonuclease IV did not contain detectable DNA phosphatase activity (<50 units/mg) when measured according to the method of Richardson (3).

Substrate Specificity—Fraction V appears to be free from endonuclease activity on intact DNA, as there was no detectable cleavage (<3%) of covalently closed circular PM2 [^{32}P]DNA after incubation with 1000 enzyme units/ml under the standard reaction conditions (Fig. 3*a*). On the other hand, the enzyme attacks PM2 DNA containing apurinic sites (Fig. 3*b*). Depurination was introduced by pH 5 treatment (11), and the treated PM2 DNA contained 0.9 to 1.0 apurinic site/molecule (Fig. 4). With 300, 600, or 1000 enzyme units/ml, 0.8 to 0.9 chain break were introduced by the enzyme in this partly depurinated PM2 DNA, while 0.4 to 0.5 chain break/DNA molecule were obtained with 100 enzyme units/ml. Further, in a different preparation of partly depurinated PM2 DNA containing 0.7 alkali-labile sites/molecule, 0.6 to 0.7 chain breaks were observed after treatment with 300 enzyme units/ml. These data indicate that endonuclease IV can make incisions at all apurinic sites in DNA, independent of sequence effects.

Endonuclease IV catalyzes the formation of single strand breaks at apurinic sites in DNA, not double strand breaks, as determined by the same methods as previously used in our characterization of a mammalian DNA endonuclease for apurinic sites (19). Phage T7 [^{3}H]DNA, containing about six apurinic sites/molecule introduced by pH 5 treatment, had a radi-

TABLE II

Requirements of endonuclease IV activity

The standard reaction mixture (0.1 ml) contains 0.2 M NaCl, 0.05 M Hepes·KOH (pH 8.2), 10^{-3} M EDTA, 10^{-4} M dithiothreitol, 50 μg of bovine serum albumin, 1 μg of depurinated alkylated [^{32}P]DNA (20,000 cpm), and 8 enzyme units.

Components added	Acid-soluble material released
	cpm
Standard reaction mixture	1440
No EDTA + 10^{-3} M $MgCl_2$	1450
No EDTA + 10^{-3} M $CaCl_2$	1410
No bovine serum albumin	900
No dithiothreitol	1320
No dithiothreitol + 10^{-3} M *p*-Hydroxymercuribenzoate	30
+ *Escherichia coli* tRNA (500 μg/ml)	1410
+ ATP (2×10^{-4} M)	1400

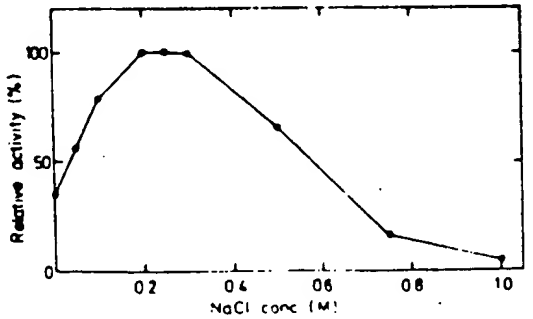

FIG. 1. Relative activity of endonuclease IV (Fraction V, 8 units) at different NaCl concentrations. The final NaCl concentration of the standard reaction mixture was varied between 0 and 1 M.

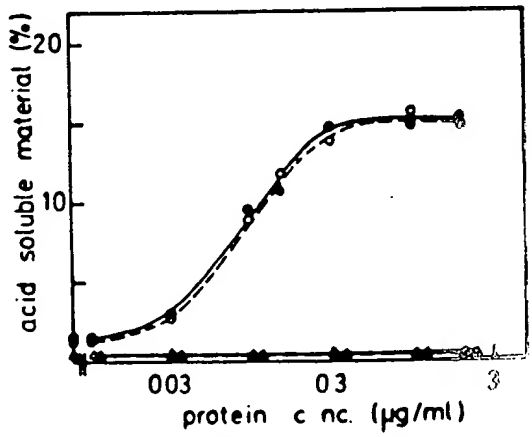

FIG. 2. Release of acid-soluble material from [^{32}P]DNA by endonuclease IV (Fraction V). ○, ●, depurinated alkylated DNA; △, ▲, native DNA; ○, △, standard assay mixture; ●, ▲, same but with 3×10^{-3} M $MgCl_2$ instead of 10^{-3} M EDTA.